REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	3-26-99	Final Report 1/15/94 - 12/31/98

4. TITLE AND SUBTITLE
Biotechnological remediation of shipboard waste effluents

5. FUNDING NUMBERS
N00014-94-1-0318

6. AUTHOR(S)
Dr. A. Konopka and Dr. R. F. Turco

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Purdue University
1392 Lilly Hall
West Lafayette, IN 47907

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Office of Naval Research
800 N. Quincy Street
Arlington, VA 22217-5000

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Biotechnological principles were applied to optimize organic matter reduction in bioreactors treating greywater effluents on Navy ships. Greywater treatment requires a rapid and reliable reduction in organic matter. Our studies addressed microbial physiological state at near-zero growth rates; this is central to the success of this approach for biotechnological remediation. The approach was to use a simulated greywater to feed a laboratory-scale bioreactor operated under conditions of 100% biomass recycle. The physiological state of the microbial population was assessed under varying operating conditions (hydraulic residence time (HRT), inputs of toxic materials, temporal changes in substrate supply, and temperature. The experimental results proved that the organic compounds present in greywater can be biodegraded by microbes very rapidly (HRT < 3 h). Surfactant biodegradation played a critical role in the performance of the bioreactor community, due to surfactant toxicity. The microbial community responded to or recovered from a variety of environmental perturbations, and maintained system performance. Many of these system properties are consequences of the unique physiological state that occurred in microbes at low substrate fluxes, in which moderate levels of biochemical activity are retained and the microbes express a broad variety of catabolic enzymes.

14. SUBJECT TERMS

15. NUMBER OF PAGES
4

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	UL

FINAL REPORT

Grant#: N00014-94-1-0318

PRINCIPAL INVESTIGATORS: Dr. A. Konopka and Dr. R.F. Turco

INSTITUTION: Purdue University

GRANT TITLE: Biotechnological remediation of shipboard waste effluents.

AWARD PERIOD: 15 January, 1994 - 31 December, 1998

OBJECTIVE: Apply biotechnological principles to optimize organic matter reduction in bioreactors treating greywater effluents on Navy ships.

APPROACH: Greywater samples obtained from the U.S. Navy was chemically analyzed to measure the amounts of different organic compounds. A simulated greywater was constructed, and used to feed a laboratory-scale bioreactor operated under conditions of 100% biomass recycle. The physiological and community responses of the microbes in the bioreactor were evaluated, with particular emphasis on determining kinetic parameters for organic matter biodegradation, and analysis of the physiological state of the microbial population existing under conditions of very low was flux per unit biomass. The laboratory scale reactor will also be used to determine the reliability of bioreactor performance to "shock loads" or other environmental perturbations likely to occur on shipboard. These include inputs of toxic materials, temporal changes in substrate supply, and temperature.

ACCOMPLISHMENTS: We conducted chemical analysis of greywater samples provided by the U.S. Navy. The organic content of the materials consisted primarily of soluble polysaccharides (about 67% by weight). Soluble protein comprised about 20%, and surfactants < 1%. We analyzed the temporal changes in the organic flux from authentic greywater and combined greywater-blackwater sources. The variability in greywater was relatively small, and thus unlikely to have substantial impact on the microbial population. There was a 10-fold fluctuation in the concentration of organic matter in the blackwater-greywater system. Particulate organic C was 20-50X higher than dissolved organic C in this wastewater feed, but surfactant levels were < 5 ppm. We used this information to construct a simulated greywater as a feed to our bioreactor.

The bioreactor was a continuous-flow system in which 100% of the biomass is retained by an ultrafiltration membrane (nominal 100,000 MW exclusion). This design has desirable characteristics from the perspectives of both engineering (low "sludge" output) and biotechnology (high biocatalyst concentration, and operation at a short hydraulic residence time (HRT)). However, it raises unanswered questions about the physiological state and stability of the microbes. Specifically, biomass accumulated to densities 50X higher than in a continuous culture (chemostat), so that the substrate flux rate per cell is 1/50 of that found in a chemostat. The physiological state and metabolic activity of individual cells is poorly understood at near-zero growth rates.

From an engineering perspective, we have demonstrated that the reactor can successfully remove the organic contaminants at HRT down to

1.5 hours, and that stable operation is maintained for at least 75 days.

From a physiological perspective, there were significant changes over the first 10 days after a shift from chemostat to biomass recycle mode. These include 2-3 fold decreases in ATP and RNA per mg microbial protein. In addition, the specific respiration rates stimulated by organic substrates decline 4-5 fold over this period. After this time, the microbes are not able to immediately initiate growth when provided with nutrients, but exhibit lag periods of 2-8 hours. This physiological state is distinct from that found in either chemostat-grown cells or starved cells. The altered state results in rates of organic metabolism and macromolecular contents that are lower than those found in more rapidly growing cells, but substantially higher than found in starved populations. Although per cell activity is low, organic additions to the bioreactor are rapidly metabolized, due to the high cell densities (> 10^{10} cells/ml). More than 80% of the organic C is catabolized to CO_2, and therefore relatively little is assimilated for biomass synthesis. The rate of biomass accumulation is twofold slower under operating conditions in which protozoa are present and capable of grazing on bacteria.

The flux of surfactants into the system is a critical factor. At high concentration, surfactants inhibit the activity of both surfactant-degrading microbes and those degrading other substrates. The system operates robustly as long as surfactant-degrading microbes maintain the surfactant concentration at a non-inhibitory concentration.

The bioreactor system can recover from inputs of toxic materials (up to 1000 mg L^{-1} hypochlorite). Although toxicants kill most of the microbes, the high biomass levels mean that there is a residual population which can rapidly regrow and reestablish system performance.

Several environmental perturbations have had no detectable impact upon bioreactor performance. The perturbations include: (a) no aeration for 24 hours (b) stopping the substrate inflow for 15 hours.

The bioreactor system is capable of degrading simulated greywater over operating temperatures from 15 to 70° C. Microbial diversity was analyzed by polymerase chain amplification of a fragment of the 16s rRNA gene and separation of products by denaturing gradient gel electrophoresis. The data showed that there was a reduction in diversity as reactor temperature was increased, and that the pattern of phylotypes changed substantially above 47° C.

The shift in the physiological state of the mixed microbial population when forced to grow at very slow rates results in a very low growth yield on the organic substrates in the waste. Whereas conventional aerobic wastewater treatment processes produce yields of 0.3 g bacterial biomass per g COD, our systems operate in the range of 0.02-0.05 g bacteria per g COD. In order to investigate the physiological mechanisms underlying these changes, a single bacterial species (Pseudomonas putida KT2442) was grown at low rates in a biomass recycle reactor, and its physiological state was compared to that of cells grown at faster rates in chemostats or batch culture, or to starved cells. The physiological characteristics at low growth rate were distinguishable from those of starved cells and cells grown at moderate growth rates. The biochemical composition (RNA and ATP content) was similar to that found in starved cells, but the potential rate of substrate catabolism (measured via respiration rates of resting cells or metabolism of radiolabeled substrate) was greater than in starved cells, but less than that found in moderately nutrient-limited cells. Cells under both severe and moderate nutrient limitations

contained broad sets of catabolic enzymes which were absent from cells growing at μ_{max}. Cells grown at very low growth rates also exhibited complex growth kinetics after a nutritional shift-up, which were not found in either starved or moderately nutrient-limited populations.

The bioreactor system is capable of degrading simulated greywater over operating temperatures from 15 to 70° C. We had previously shown that microbial diversity in the system decreased as temperature was increased. We have also found that the physiological characteristics of the system are affected by temperature. Although the microbial community present at moderately thermophilic temperatures is capable of metabolic activity over a temperature range of 25-30° C, the populations at higher temperatures exhibit a higher maintenance energy requirement, and therefore are more susceptible to interruptions in substrate supply than mesophilic reactors.

The physiological heterogeneities of individual cells was analyzed by measurement of the RNA content in cells by fluorescence microscopy after staining with the fluorochromes Hoechst 33342 (binds to DNA) and pyronin Y. The distribution of cell RNA content was approximately Gaussian, either at quasi-steady state, or after a nutritional shift-up. This was true either for the mixed microbial population in the bioreactor, or for individual species (Escherichia coli and Arthrobacter sp.).

The degradation of surfactants in greywater is hypothesized to be the limiting step in the operation of these bioreactors. Attempts are being made to isolate the microbe or consortium of microbes capable of degrading the surfactant, linear alkylbenzenesulfonate (LAS). A technical problem is that LAS is toxic even to the microbes which degrade it; therefore, it is difficult to obtain cultures with a high cell density for physiological and biochemical studies. Attempts were made to circumvent this problem via the addition of auxiliary substrates to continuous flow reactors in which LAS was a carbon source. These attempts were unsuccessful, although they did lead to selection of microbes which were resistant to LAS concentrations of 1-2 mM (whereas in our typical reactors, LAS is toxic to microbes at 150 micromolar). By using LAS as sole carbon source, we have produced a low cell density system which degrades LAS, and contains 6 bacterial types. This consortium was characterized both phylogenetically and phsyiologically.

CONCLUSIONS: The organic compounds present in graywater can be biodegraded by microbes very rapidly, such that bioreactors could operate at HRT < 3 h. The biodegradation of surfactants plays a critical role in the performance of the bioreactor community, because these molecules are toxic to microbes at high concentration. The microbial community can respond to or recover from a variety of environmental perturbations, and maintain system performance. Many of these system properties are consequences of the unique physiological state that occurs in microbes at low substrate fluxes, in which moderate levels of biochemical activity are retained and the microbes express a broad variety of catabolic enzymes.

SIGNIFICANCE: The shipboard treatment of greywater effluents requires a rapid and reliable reduction in organic matter. Microbial degradation has been shown to be useful because it can be done under conditions which are rapid, reliable, and with minimal sludge production. Our studies have addressed microbial physiological state at near-zero growth rates, which is central to determining the success of this approach for biotechnological remediation.

PUBLICATIONS AND ABSTRACTS (for total period of grant):

1. Konopka, A. (1995) Biotechnological remediation of shipboard waste effluents. Proceedings of US-Japan Technical Workshop on Shipboard Wastes Treatment Technology.

2. Konopka, A. (1995) Microbial physiology of wastewater treatment in a biomass-recycle reactor. Abstr. Soc. Gen. Microbiol., Aberdeen.

3. Oliver, L., Zakharova, T., Konopka, A. and Turco, R.F. (1996) Sodium hypochlorite perturbation of a graywater treatment system. Abstr. Annu. Mtg. Amer. Soc. Microbiol.

4. Oliver, L., Zakharova, T., Konopka, A. and Turco, R.F. (1996) Sodium hypochlorite perturbation of a graywater treatment system. 51st Industr. Waste Conf., Purdue Univ.

5. Konopka, A., T. Zakharova, L. Oliver, D. Camp and R. F. Turco. 1996. Biodegradation of organic wastes containing surfactants in a biomass recycle reactor. Appl. Environm. Microbiol 62: 3292-3297.

6. Konopka, A. and Zakahrova, T. (1997) Physiological state at very low growth rate. Abstr. Annu. Meeting Amer. Soc. Microbiol.

7. Konopka, A., T. Zakharova, L. Oliver, and R. F. Turco. 1997. Microbial biodegradation of graywater in a continuous-flow reactor. J. Industr. Microbiol. Biotechnol. 18:235-240.

8. Konopka, A., T. Zakharova, E. Paseuth, L. Oliver, and R. F. Turco. 1998. Physiological state of a microbial community in a biomass recycle reactor. J. Industr. Microbiol. Biotechnol 20: 23-237.

9. Konopka, A., L. Oliver, and R.F. Turco. 1998. The use of carbon substrate utilization patterns in environmental and ecological microbiology. Microbial Ecology 35: 103-115.

10. Oliver, L., A. Konopka, T. Zakharova, and R. Turco. Sodium hypochlorite perturbation of a graywater treatment system. Submitted to Water Sci. and Technol.

11. Konopka, A. and T. Zakharova. Physiological state of Pseudomonas putida KT2442 at slow growth rates. Submitted to Arch Microbiol

12. Konopka, A. T. Zakharova, and T. LaPara. Bacterial function and community structure in reactors treating biopolymers and surfactants at mesophilic and thermophilic temperatures. Submitted to J. Industr. Microbiol. Biotechnol.